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STRUCTURAL ALTERATIONS OF TRANSFORMING GROWTH FACTOR-β RECEPTOR GENES IN HUMAN CERVICAL CARCINOMA

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The development and progression of invasive uterine cervical carcinomas appear to be associated with the progressive loss of sensitivity to transforming growth factor-β (TGFβ)-mediated cell cycle arrest. In order to identify possible molecular mechanisms responsible for TGFβ resistance, we screened the 7 exons of the type II (*TβR-II*) TGFβ receptor and the 9 exons of the type I (TβR-I) TGFβ receptor genes for mutations in 16 paraffin-embedded primary invasive cervical carcinoma specimens. In one of these carcinomas, we found a novel G→T transversion in exon 3 of *TβR-II* that introduces a premature stop codon (E142Stop) and presumably results in the synthesis of a truncated soluble exoreceptor. In one tumor, a silent A→C transversion mutation that may affect mRNA splicing was present in exon 6 of *TβR-I*. In addition, 7 of 16 cases were heterozygous for a G→A polymorphism in intron 7 of *TβR-I*. Finally, we identified a 9 base pair in-frame germline deletion in exon 1 of *TβR-I* resulting in loss of 3 of 9 sequential alanine residues at the N-terminus in 6 of 16 cases. Analysis of specimens from case-control studies indicated that carriers of this del(GGC)$_3$ *TβR-I* variant allele may be at a increased risk for the development of cervical carcinoma ($p=0.22$). Furthermore, the response of cells expressing the variant receptor to TGFβ was diminished. Our results support the notion that diverse alterations in the TGFβ signaling pathway may play a role in the development of cervical cancer. *Int. J. Cancer* 82:43–51, 1999.
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Carcinomas of the uterine cervix are among the most common malignancies of women worldwide (Parker *et al*., 1998). A relatively unique aspect of cervical cancer (CC) is that the initial transforming event involves infection of normal cervical epithelial cells with so-called high-risk human papillomaviruses (HPV) (reviewed by Howley, 1991). Most precancerous cervical lesions termed cervical intraepithelial neoplasia (CIN) and almost all malignant lesions contain HPV DNA. Two viral transforming proteins, E6 and E7, confer immortalization and loss of cell cycle control by inactivating 2 key cellular regulators of apoptosis and cell cycle progression: p53 and the retinoblastoma protein, pRb, respectively (reviewed by Howley, 1991). Prospective clinical studies have demonstrated that only a small fraction of early cervical dysplasias progress to severe dysplasia, while the risk of progression to invasive CC is much greater for severely dysplastic lesions (Koss *et al*., 1963). This raises the important question of which genetic or biological characteristics distinguish CIN lesions that will progress to cancer from the majority that spontaneously regress.

A particularly interesting biological characteristic associated with malignant progression of cervical epithelial cells is their progressive loss of responsiveness to TGFβ (Braun *et al*., 1990; Creek *et al*., 1995; De Geest *et al*., 1994; Kang *et al*., 1998; Kim *et al*., 1998). TGFβ belongs to a multifunctional family of growth factors that tightly regulate basic cellular functions such as proliferation, differentiation and extracellular matrix turnover. In HPV-immortalized cervical epithelial cells, TGFβ inhibits transcription of the HPV16 E6 and E7 genes and blocks G_1 to S-phase cell cycle progression (Woodworth *et al*., 1990). TGFβ-dependent repression of E6 and E7 transcription appears to be mediated by a TGFβ-response element (TRE) located within the principal viral promoter and enhancer region (long control region, LCR) (Canhoto *et al*., 1997). Primary cervical keratinocytes that are immortalized by HPV in vitro and are passaged in culture for prolonged periods of time, eventually lose their sensitivity to the inhibitory effects of TGFβ1 (Creek *et al*., 1995; De Geest *et al*., 1994). In these TGFβ-resistant cells, transcription of the E6 and E7 genes is no longer repressed by TGFβ (Creek *et al*., 1995). In addition, some cell lines derived from CIN lesions are sensitive to TGFβ, whereas lines derived from invasive CCs are resistant (Braun *et al*., 1990; De Geest *et al*., 1994; Kang *et al*., 1998; Kim *et al*., 1998; Woodworth *et al*., 1990). Thus, these in vitro results suggest a strong association between the loss of TGFβ1-mediated repression of the *HPV E6/E7* genes and the transition from precursor lesions to invasive CC. Integration of the HPV genome within the host cell genome is associated with disruption of the viral *E2* gene, resulting in the deregulated expression of the *E6* and *E7* transforming genes. However, studies have suggested that the TRE located within the viral LCR is retained (Canhoto *et al*., 1997). Thus, the loss of TGFβ-dependent regulation of *E6/E7* gene transcription in invasive CC is more likely due to inactivation of the TGFβ signaling pathway itself than to deletion of TGFβ-responsive elements within the viral genome.

The TGFβ signal is transduced by a pair of transmembrane serine-threonine receptor kinases (reviewed by Heldin *et al*., 1997). TGFβ1 binds primarily to the TGFβ type II receptor (TβR-II), followed by the recruitment of the TGFβ type I receptor (TβR-I) into a heterotetrameric complex with 2 TβR-II molecules and a single ligand dimer. Upon formation of this ternary complex, the TβR-II kinase phosphorylates specific serine residues immediately upstream of the serine-threonine kinase of TβR-I, which results in activation of the TβR-I kinase (Heldin *et al*., 1997). Two mammalian homologues of the Drosophila mother-against-dpp (Mad) protein, Smad2 and Smad3, then become transiently associated with and phosphorylated by the activated TβR-I receptor. This results in the formation of a heterotetrameric complex with a third member of the Mad superfamily, Smad4. This complex is then translocated to the nucleus where it binds to DNA and regulates transcription of specific target genes (Heldin *et al*., 1997).

Kang *et al*. (1998) examined the expression and structural integrity of the *TβR-I* and *-II* genes in a series of 8 human CC cell lines. Two of these lines failed to express TβR-II-specific mRNA, which was due to a homozygous deletion of the gene in 1 of the 2 cases (Kang *et al*., 1998).

In addition, missense mutations, gross gene rearrangements and truncated or decreased transcripts of the *TβR-II* gene have been found in a variety of tumor types (reviewed by Reiss, 1997).

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Moreover, loss of *TβR-I* mRNA and/or protein expression appears to occur frequently in pancreatic carcinomas, renal cell cancer cell lines and chronic lymphocytic leukemia (DeCoteau *et al*., 1997; Lagneaux *et al*., 1997; Ramp *et al*., 1997). In addition, mutations of the *TβR-I* gene have been described in primary and metastatic breast carcinomas (Chen *et al*., 1998). Thus, inactivation of both *TβR* genes occurs in subsets of human tumors.

Because of the strongly suggestive evidence that cervical neoplasia is associated with loss of TGFβ responsiveness, we have begun to investigate the status of the TGFβ signaling pathway in cervical cancer. We now report a comprehensive structural analysis of the TβR-I or -II genes in a series of human CC specimens. We discovered a novel mutation in the *TβR-II* gene as well as several novel polymorphisms in the *TβR-I* gene, one of which may predispose for the development of CC.

MATERIAL AND METHODS

Tissue specimens and DNA extraction

Matching normal and tumor paraffin-embedded tissues were obtained from 16 patients with invasive carcinomas of the uterine cervix treated at the University of Groningen, The Netherlands. There were a total of 14 patients with squamous cell carcinomas and 2 patients with a poorly differentiated (''glassy cell'') adenocarcinoma. Genomic DNA was extracted from tumor and normal tissues as previously described (Chen *et al*., 1998). Isolating genomic DNA from a single 5μm microdissected paraffin-embedded tumor section using InstaGene matrix (Bio-Rad, Hercules, CA) typically yields 200μl of DNA template solution. Adjacent tumor sections were reviewed to confirm the histological diagnosis and to ensure that at the majority of the cells in the specimen were carcinoma cells.

Genotyping of TGFβ signaling intermediates

We optimized our experimental conditions to generate sufficient amounts of PCR product from 5μl aliquots of template DNA to conduct single-strand conformation polymorphism (SSCP) analysis of each of the 7 exons of TβR-II, and each of the 9 exons of TβR-I using AmpliTaq Gold DNA polymerase (PE; Applied Biosystems, Foster City, CA). Primers used to amplify TβR-II exons were those previously described by Lu *et al.* (1996). These included intronic primers flanking each of the intron-exon boundaries, as well as 6 internal primers to amplify the large exon 4. Each of the 9 exons of the TβR-I gene was amplified using the flanking intronic primer pairs as previously described (Chen *et al.*, 1998).

The *TβR-II* and -I genes were analyzed by PCR-SSCP as previously described (Chen *et al*., 1998). Suspect bands were excised from the gels, reamplified, and subjected to DNA sequencing as previously described (Chen *et al*., 1998). The presence of any sequence alteration was always confirmed by repeated PCR-SSCP and sequencing using an independent aliquot of tumor-derived genomic DNA as starting material. Whether any mutations were somatic in nature or present in the germline was determined by analyzing genomic DNA isolated from normal tissue of the same patient.

Case-control studies

We examined the epidemiologic association between CC and the germline TβR-I genotype in 2 different case-control studies. In the first study, subjects were randomly selected from among participants in a larger NCI-sponsored multicenter case-control study of in situ and invasive CC of various histological types conducted at 6 clinical centers in the eastern United States. The patients selected for our study (n=37; median age: 40, range: 27–66) had histologically confirmed invasive squamous cell carcinoma of the cervix. Controls (n=38; median age: 37, range: 21–71) were selected to be representative of the cases with respect to age, ethnicity and geographical location. Genomic DNA from all subjects was extracted from exfoliated cervical cells stored in STM buffer (Digene, Beltsville, MD). The majority of patients were sampled following resection of their primary tumor and a confounding effect on our findings of contamination of the samples with tumor cells could be excluded.

In the second study, we examined invasive CC cases (n=29; median age 38, range: 22–51), enrolled through institutional affiliates of the University of the West Indies Hospital in Kingston, Jamaica, between November 1994 and April 1998. Controls (n=30; median age: 34.5, range: 24–66) were sequential cervical cytology screening clinic patients with normal current Pap smears and self-reported history of past normal Pap smears, enrolled through the university-affiliated clinic (Spring 1996 and 1997), and then frequency age-matched to cases. All subjects in this study were of African descent and resided in the Kingston area. We isolated germline genomic DNA for PCR by punching out 3 mm circles from dried blood blots using Acu-Punch skin biopsy punches (Acuderm, Ft. Lauderdale, FL). Specimens were then processed using the dried blood kit (BioRad) according to the protocol provided by the manufacturer, and subjected to PCR for *TβR-I* genotyping.

Vectors used for transfection

The pHA-1 mammalian expression vector was constructed by subcloning the full-length human *TβR-I* (ALK-5, (Franzén *et al*., 1993)) into the expression vector, pCDNA3 (Stratagene, La Jolla, CA) thereby placing it under transcriptional control of a cytomegalovirus (CMV) promoter. To facilitate detection and quantitation of transfected receptor, the influenza virus HA epitope tag YPYDVPDYA was introduced at the COOH-terminus of the protein. Deletion mutants of *TβR-I* were constructed by synthesizing oligonucleotides that lacked 3, 6 or all 9 GGC trinucleotide repeats and flanking sequences of TβR-I exon 1, and subcloning the resulting Nco1-Sma1 fragment into a previously constructed pGEM vector containing the 5' half of wild type *TβR-I* (pGEM R/B). An EcoR1-Xho1 fragment excised from this modified pGEM R/B was then subcloned into a pHA-1 backbone, which had been prepared by partial digestion of pHA-1 with EcoR1/Xho1.

Reporter gene assays

The signaling capability of wild type and mutant *TβR-I* receptors was assessed in transient transfection assays into R-1B/L17 cells, a subclone of Mv1Lu mink lung epithelial cells (A generous gift of Dr. J. Massagué, New York, NY). This cell line is convenient because it is refractory to TGFβ, fails to express detectable levels of TβR-I, and all responses to TGFβ can be restored by re-expressing wild type *TβR-I*. The firefly luciferase reporter gene construct, pSBE4 (a generous gift from Dr. B. Vogelstein, Baltimore, MD), in which 4 tandem repeats of a Smad4-specific DNA binding element (SBE) drive the luciferase cDNA, was used to assess cellular responsiveness to TGFβ (Zawel *et al*., 1998). Transfections were carried out using Lipofectin (GIBCO-BRL, Gaithersburg, MD) as previously described (Chen *et al*., 1998). In order to control for variations in transfection efficiency, we co-transfected a small amount (0.01μg) of pRL-CMV, a plasmid expressing a *Renilla luciferase* reporter gene (Promega, Madison, WI). Cell lysate was mixed with the appropriate luciferase assay reagent and photon emission was measured using a Series 20 Barthold Luminometer (Turner Designs, Sunnyvale, CA).

Receptor expression and subcellular localization

Cultures were washed twice with ice cold PBS, and cells were collected by scraping with a rubber policeman into a hypotonic solution of 1.5 mM $MgCl_2$. After incubation on ice for 30 min, cells were disrupted by brief sonication, and the membrane fraction was isolated by centrifugation at 12,500g for 20 min at 4°C. The supernatant (cytosolic fraction) was set aside and membrane proteins were solubilized in buffer containing 1% [v/v] Triton X-100, 0.1% [w/v] SDS, 150 mM NaCl, 50 mM Tris [pH 7.5], 3 mM sodium azide, 1 mM PMSF and 2μg/ml leupeptin. After boiling for 10 min in the presence of sample buffer, aliquots containing equal amounts of cytosolic and membrane protein were

resolved by electrophoresis on a 10% (w/v) SDS-polyacrylamide gel and subjected to Western immunoblotting by using rabbit polyclonal antiserum directed against the HA peptide (HA.11, BAbCO, Richmond, CA.). Blots were developed using horseradish peroxidase-tagged goat anti-rabbit IgG and the bands visualized using DuPont NEN (Boston, MA) Chemiluminescence Reagent as recommended by the manufacturer.

Statistical analysis

Dichotomous and ordered data were organized into contingency tables and analyzed by Pearson chi-square, Fisher's exact test (when expected values were <5) or by the Mantel extension test to measure trends. Prevalence rates were estimated and 95% confidence intervals (95% CI) around each estimate were determined assuming a binomial distribution. For comparisons of continuous values (*e.g.*, pSBE4-Luc activity), the Student *t*-test was used.

RESULTS

In order to identify a possible molecular basis for TGFβ-resistance in CC, we conducted a comprehensive structural analysis of both the *TβR-I* and *-II* genes in 16 invasive cancers of the uterine cervix (14 squamous cell carcinomas and 2 adenocarcinomas) (Table I). Fifteen specimens were derived from primary tumors, and one from an abdominal wall metastasis. Each of the 7 exons and flanking splice sites of the TβR-II gene were analyzed by PCR-SSCP. The only abnormality we encountered was an aberrant band in exon 3 in the case of tumor CT59. DNA from the shifted band was reamplified and subjected to direct sequencing (Fig. 1*a*). This revealed a G→T transversion at nucleotide 425 (GAG to TAG) which converts the glutamic acid residue at position 142 to a stop-codon (E142Stop) (Fig. 1*a*). This is the first such mutant described in any human neoplasm. Based on the location of the stop codon, this mutant presumably encodes a truncated TβR-II protein that is not anchored in the cell membrane and is presumably released into the extracellular milieu (Fig. 1*b*).

In contrast with TβR-II, we encountered several different structural alterations of the TβR-I gene (Table II). First, we identified a single tumor (CT65) with a somatic A→C transversion at nucleotide 1125 (codon 375, ACA→ACC) located in exon 6 of *TβR-I* (Fig. 2). Although this mutation does not alter the amino acid sequence of the protein, it is located at position -6 from the exon-intron boundary and may affect RNA splicing. Secondly, we identified a nucleotide (G→A) substitution within intron 7 of TβR-I (Fig. 3). This G→A transition appears to represent a polymorphic allele as 7 of 16 cases (44%, 95% CI: 20–70%) of both germline and tumor DNA were heterozygous. We took advantage of the fact that this sequence change abolishes a BsrI restriction site to estimate the allele frequency of this variant sequence in a larger U.S. population. By using PCR and BsrI restriction digestion of germline DNA to distinguish between the wild type and variant alleles, we found that 24 of 38 randomly selected individuals were homozygous for the wild type sequence (63%, 95% CI: 46–78%), 12 were heterozygous (32%, 95% CI: 17–49%), and 2 were homozygous for the variant sequence (5%, 95% CI: 1–18%). These genotype frequencies were very similar to those found among the 16 patients with CC from the Netherlands (Fisher's exact test, $p=1.000$).

Perhaps our most important finding is a germline variant of the *TβR-I* gene which gives rise to a distinct SSCP pattern (Fig. 4*a*) and which lacks 3 of 9 consecutive GGC repeats located within exon 1 (Fig. 4*b*). Six of 16 patients were heterozygous carriers of this del$(GGC)_3$ *TβR-I* variant (37.5%, 95% confidence interval [CI]: 15–65%). Interestingly, the allele frequency of the del$(GGC)_3$ variant among our CC population (18.8%, 95% CI: 7–36%) was

TABLE I – CHARACTERISTICS OF CARCINOMAS OF THE UTERINE CERVIX AND *TβR* GENE[1] ALTERATIONS

Case	Histology	Stage	Follow-up	*TβR-I*	*TβR-II*
CT51	Poorly differentiated adeno-carcinoma	IIb	NED	Exon 1 del$(GGC)_3$ Intron 7 variant	WT
CT52	Moderately differentiated SCC	Ib	NED	Intron 7 variant	WT
CT53	Poorly differentiated SCC	Ib2	NED	WT	WT
CT54	Moderately differentiated SCC	IIb	NED	WT	WT
CT55	Well-differentiated SCC	Ib2	NED	WT	WT
CT56	Well-differentiated SCC	IIa	NED	WT	WT
CT57	Poorly differentiated SCC	Ib1	NED	Exon 1 del$(GGC)_3$	WT
CT58	Poorly differentiated adeno-carcinoma	Ib1	NED	Exon 1 del$(GGC)_3$ Intron 7 variant	WT
CT59	Moderately differentiated SCC	NA	Lost to follow up	WT	E142Stop
CT60	Moderately differentiated SCC	NA	Lost to follow up	Intron 7 variant	WT
CT61	Poorly differentiated SCC	IVb	Peritoneal implants at 2 months	WT	WT
CT62	Abdominal wall SCC metastasis	IV	NED	Exon 1 del$(GGC)_3$	WT
CT63	Moderately differentiated SCC	IIa	NED	Intron 7 variant	WT
CT64	Poorly differentiated SCC	Ib1	NED	WT	WT
CT65	Moderately differentiated SCC	Ib1	NED	Exon 1 del$(GGC)_3$ Exon 6 a1 125c Intron 7 variant	WT
CT66	Moderately differentiated SCC	Ib2	Local recurrence at 5 months	Exon 1 del$(GGC)_3$ Intron 7 variant	WT

[1]Genomic DNA was extracted from 5 μm thick paraffin sections from 16 cervical carcinoma specimens. Each of the 7 exons of *TβR-II* and the 9 exons of *TβR-I* were screened for the presence of mutations by PCR-SSCP as described in Material and Methods. Suspect bands were excised, and the DNA re-amplified and subjected to direct sequencing. The presence of individual DNA sequence alterations was confirmed by applying the entire procedure to a second aliquot of genomic DNA. In each of the 9 cases in which we identified either the del$(GGC)_3$ or the intron 7 variant *TβR-I* alleles, both germline and tumor DNAs were heterozygous. NED: No evidence of disease. WT: Wild type. SCC: Squamous cell carcinoma.

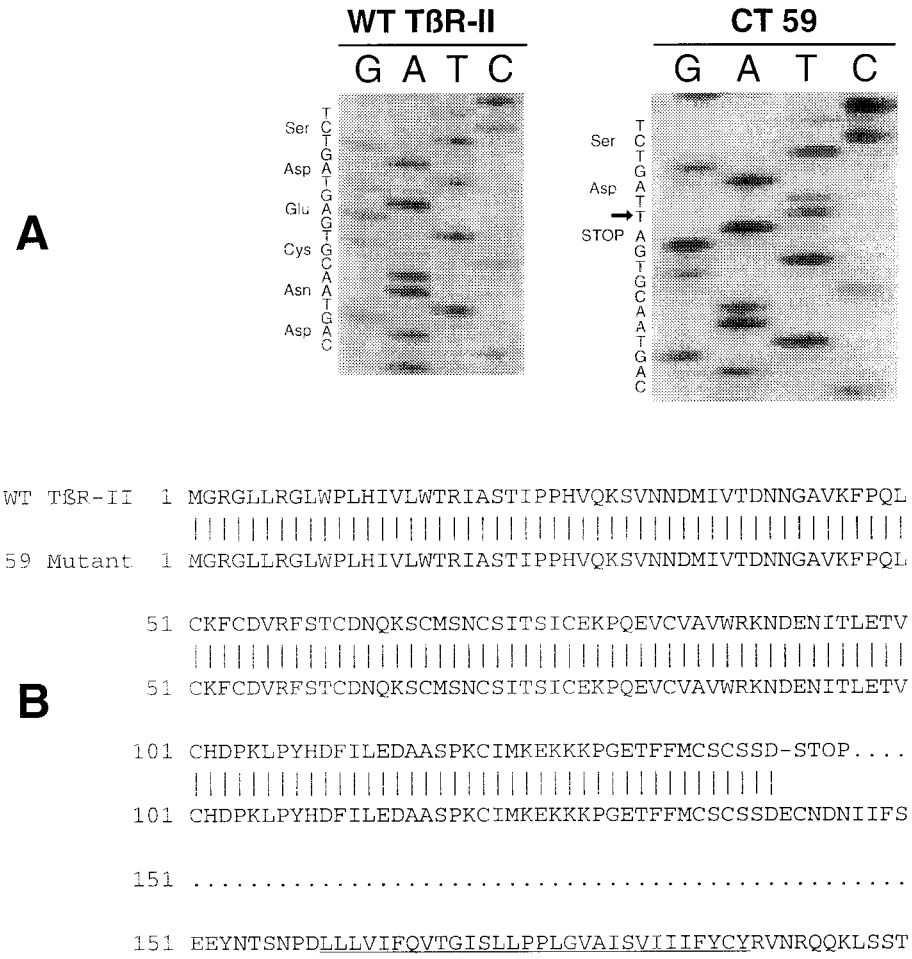

FIGURE 1 – Nonsense mutation in 5' half of *TβR-II* gene. (*a*) Partial DNA sequence of exon 3 of *TβR-II* in tumor CT 59 revealed a G→T transition (nucleotide 425 from ATG). This mutation introduces a premature stop codon at position 142. (*b*) Amino acid sequence comparison between the N-terminal half of wild type and CT 59 mutant *TβR-II*. The transmembrane domain is underlined.

significantly higher than among normal blood donors as reported by Pasche *et al*. (1998) (4.2%). This discrepancy suggested the possibility that the del$(GGC)_3$ TβR-I variant might confer a susceptibility for the development of CC. In order to address this important question, we compared the frequencies of TBR-I genotypes among subjects culled from 2 different recently completed case-control studies. In the first study, 8 (22%; 95% CI: 10–38%) of 37 CC cases were heterozygous carriers of the del$(GGC)_3$ variant of TBR-I, as compared with 4 (11%; 95% CI: 3–25%) of 38 control subjects. In addition, one case but no controls was homozygous for the del$(GGC)_3$ variant (Table II*a*). The overall trend to these data was of borderline significance (Mantel extension test for trend; $p=0.15$). In the second study, which was conducted amongst Jamaican women of African descent, 3 (10%; 95% CI: 2–27%) of 29 cases and 3 (10%; 95% CI: 2–26%) of 30 controls were heterozygous carriers of the variant allele (Table II*b*). Thus, an association between the del$(GGC)_3$ variant of *TBR-I* and development of CC was observed in U.S. but not in Jamaican women. The explanation for this is unknown, as small sample size and a true biologic difference equally fit the data. We note that the prevalence among controls was similar in U.S. and Jamaican subjects. Therefore, we re-examined the results from the 2 populations as if they were derived from 2 separate strata within a single investigation and measured the overall association of the del$(GGC)_3$ variant with CC (Table II*c*). The non-significant (Mantel extension test, summary chi-square = 1.5; $p=0.22$) but positive trend in the data suggests that there may be a true biological difference between the 2 study populations. The notion that carriers of the del$(GGC)_3$ allele may be at higher risk for the development of CC is further supported by the fact that only 1 of 7 squamous cell carcinoma cell lines derived from tumors of the upper aero-digestive tract carried the del$(GGC)_3$ allele, whereas 1 of 2 cervical carcinoma lines (CaSki) was heterozygous for the variant allele (data not shown).

We studied the biological effects of the 3-alanine deletion in the del$(GGC)_3$ variant in transient transfection assays by using the TβR-I-deficient R-1B (L17) mink lung epithelial cell line. Activation of the Smad DNA-binding element (SBE) in pSBE4-Luc reflects TGFβ-induced gene transcription (Zawel *et al*., 1998). In R-1B (L17) cells transfected with wild type *TβR-I*, TGFβ-mediated pSBE4-dependent luciferase activity was increased approximately 5.5-fold over controls, while the increase observed in cells transfected with the del$(GGC)_3$ mutant was only approximately 4-fold ($p=0.0023$, Student *t*-test) (Fig. 5*a*). Because of the possibility that some of the phenotypic differences between wild type and del$(GGC)_3$ *TβR-I* might be masked because of the high levels of expression induced by the strong CMV promoter, we also examined the effects of reducing the level of expression by transfecting smaller amounts of *TβR-I* DNA. As shown in Figure 5*b*, pSBE4-Luc activity was consistently lower in cells expressing the del$(GGC)_3$ variant compared to wild *type TβR-I*, with the difference being most pronounced when the smallest amounts of *TβR-I* DNA were transfected ($p=0.023$, Student *t*-test for paired observations).

TABLE II – *TβR-I* GENOTYPES AND CASE-CONTROL STUDIES

Genotype	Cases	Controls	Odds Ratio
A. U.S. study[1]			
Homozygous WT	29	34	1.00
Heterozygous	7	4	2.05
Homozygous del$(GGC)_3$	1	0	Infinity
All	37	38	
Genotype	**Cases**	**Controls**	**Odds Ratio**
B. Jamaican study[2]			
Homozygous WT	26	27	1.00
Heterozygous	3	3	1.04
Homozygous del$(GGC)_3$	0	0	N/A
All	29	30	
Genotype			**Weighed Odds Ratio**
C. Overall association of the del$(GGC)_3$ variant with CC[3]			
Homozygous WT			1.00
Heterozygous			1.59
Homozygous del$(GGC)_3$			—[4]

[1]Chi-square test for independence; chi-square: 2.202; $p = 0.33$. Mantel extension test for trend: chi-square: 2.072; $p = 0.15$.–[2]Fisher's exact test: $p = 0.96$. Mantel extension test for trend: chi-square: 0.002; $p = 0.97$.–[3]Mantel extension test for trend, treating U.S. and Jamaican subjects as 2 separate, equally weighted strata within a single investigation (Summary chi-square = 1.5; $p = 0.22$).–[4]One U.S. case but no Jamaican subjects or U.S. controls were homozygous for the del$(GGC)_3$ variant. CI: Confidence interval. WT: Wild type. N/A: Not applicable. A: Patients with invasive squamous cell carcinoma of the cervix (n = 37; median age: 40, range: 27–66) were randomly selected from among participants in a NCI-sponsored multicenter case-control study of *in situ* and invasive CC conducted at 6 clinical centers in the eastern United States. Controls (n = 38; median age: 37, range: 21–71) were selected to be representative of the cases with respect to age, ethnicity, and geographical location. B: In the Jamaican study, patients of African decent with invasive CC (n = 29; median age: 38, range: 22–51) were enrolled through institutional affiliates of the University of the West Indies Hospital in Kingston, Jamaica, between November 1994 and April 1998. Controls (n = 30; median age: 34.5, range: 24–66) were sequential cervical cytology screening clinical patients with normal current pap smears and self-reported history of past normal Pap smears, enrolled through the university-affiliated clinic (spring 1996 and 1997), and then frequency age-matched to cases. C: As the prevalence of the variant *TβR-I* allele in the 2 control populations was not significantly different (5.3%, 95% CI: 1–13 in study A, 5.0%, 95% CI: 1–14 in study B), it is legitimate to analyze the data from the 2 studies as a single investigation with two strata using the Mantel extension summary test. The *p*-value of 0.22 suggests that a true biological difference might exist between the 2 populations with regards to the effects of the del$(GGC)_3$ *TβR-I* variant.

The in-frame deletion of 3 GGC repeats in del$(GGC)_3$ encodes a protein that lacks 3 of 9 consecutive alanine residues. Based on its location, it was possible that this alanine repeat sequence is part of the putative TβR-I signal peptide (Fig. 4*c*) (Franzén *et al*., 1993). This idea is supported by comparative hydrophobicity plots of wild type and the del $(GGC)_3$ *TβR-I* variant that predict that the deletion shortens the putative hydrophobic core of the signal peptide (Fig. 4*c*). As a consequence, the deletion might affect the ability of the TβR-I protein to be targeted to the cell membrane. Alternatively, the 9 alanines may be located at the extreme N-terminal end of the mature protein and be involved in ligand binding. In order to address these questions, we constructed a series of in-frame deletion mutants of TβR-I that lacked either 3, 6 or all 9 of the GGC repeats [del$(GGC)_3$, del$(GGC)_6$, or del$(GGC)_9$]. To determine whether the 9 alanines at the N-terminus of *TβR-I* were required for membrane localization, cytosolic and cell membrane preparations of cells transfected with the del$(GGC)_3$, del$(GGC)_6$ or del$(GGC)_9$ deletion mutants were subjected to Western immunoblotting using anti-HA monoclonal antibody (Fig. 5*c*). In all cases, almost all of TβR-I protein was found in the membrane fraction. In addition,

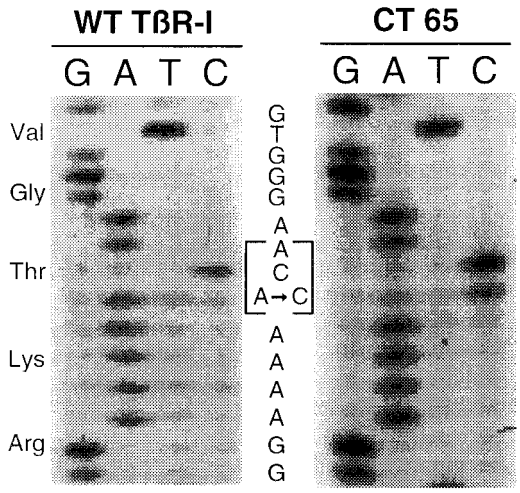

FIGURE 2 – Silent mutation in exon 6 of TβR-I. Partial DNA sequence of exon 6 of *TβR-I* found in tumor CT65 revealed a A→C transversion (nucleotide 1125 from ATG) which does not alter the amino acid sequence, but may affect RNA splicing.

there were no significant differences in the amounts of membrane-associated receptor between cells transfected with wild type and any of the mutant *TβR-I* forms (Fig. 5*c*). Thus, the 9 alanine repeat sequence is apparently not required for expression of TβR-I at the cell membrane, but must play a different role in TGFβ signaling.

In summary, cells expressing the del$(GGC)_3$ variant were significantly less sensitive to the effects of TGFβ on gene transcription than cells expressing the wild type receptor. It is possible that these functional differences form the biological basis for the increased susceptibility to cervical carcinoma.

DISCUSSION

We have systematically investigated TGFβ signaling intermediates in human CC. The status of both the *TβR-I* and *-II* genes was compared in the same human tumor samples. We made several interesting and potentially important observations. With regards to the *TβR-II* gene, we identified a novel G→T transition mutation at position 425, which introduces a premature stop codon (E142Stop) within the 5' half of the gene. Interestingly, nucleotide 425 is located 40 nucleotides downstream of the polyadenine tract in which most of the frameshift mutations in colorectal and gastric cancers of individuals with DNA mismatch repair deficiencies occur (Markowitz *et al*., 1995). Based on its location, we presume that the E142Stop mutant encodes a truncated and soluble TβR-II exoreceptor. Lin *et al*. (1995) have shown that such soluble TβR-II exoplasmic domain receptors bind TGFβ1 and TGFβ3 with high affinity and effectively compete for binding of TGFβ to cell surface receptors thereby protecting cells against TGFβ-mediated growth arrest in a dominant-negative fashion. Moreover, our finding of a novel nonsense mutation at nucleotide 425 of *TβR-II* is in agreement with previous studies suggesting that malignancy-associated mutations of the *TβR-II* gene are clustered either in the extracellular domain or in the C-terminal portion of the serine-threonine kinase (reviewed by Reiss, 1997). The fact that we found only a single case with a *TβR-II* mutation indicates that CC is similar to other tumor types in that the frequency of mutational inactivation of the TβR-II gene is low (Reiss, 1997). Kang *et al*. (1998) reported a single CC cell line that had undergone a homozygous deletion of the *TβR-II* gene, while the structural integrity of this gene was retained in the remaining 7 CC. These observations further strengthen the impression that inactivation of *TβR-II* occurs in only a minority of CC cases.

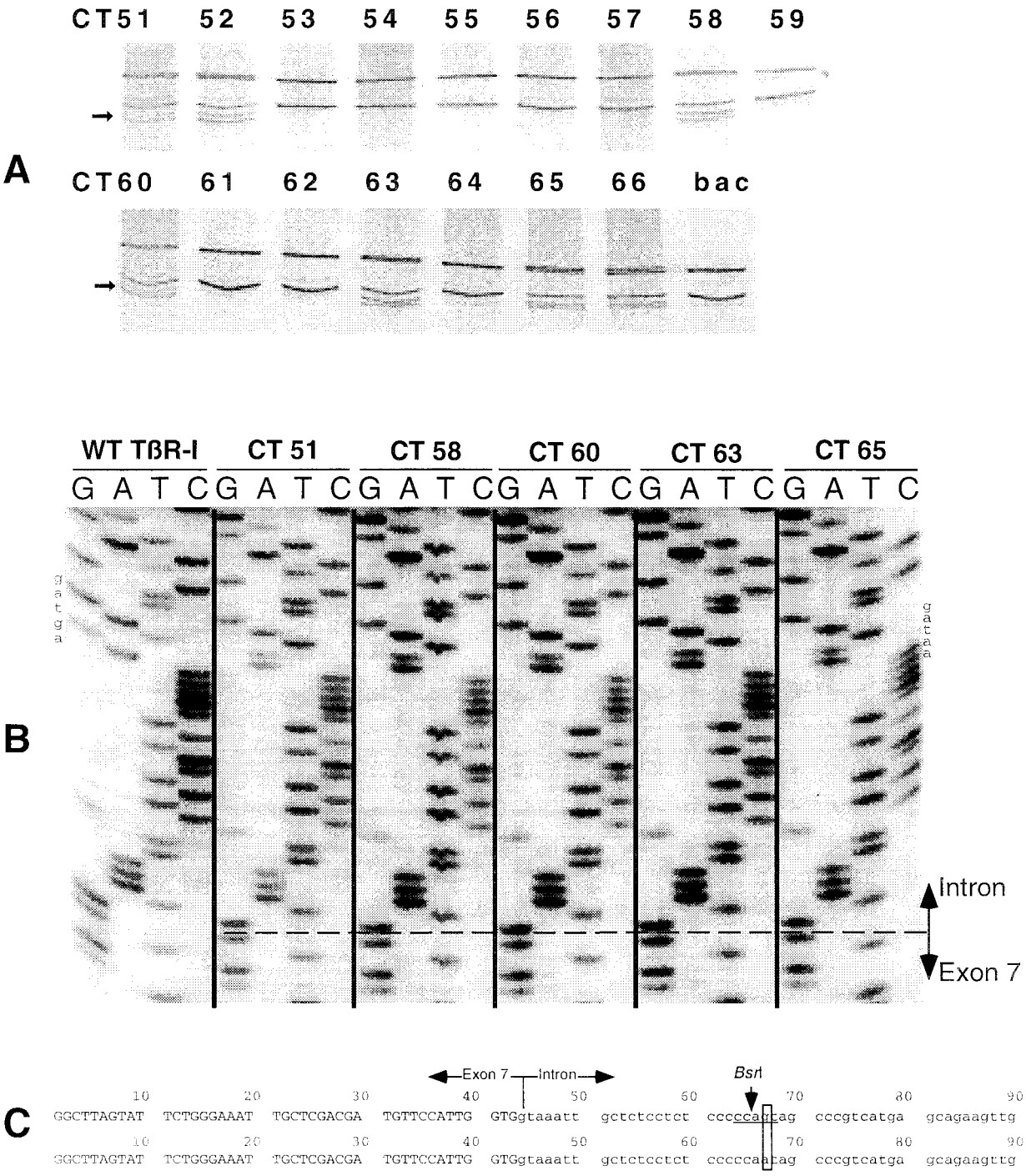

FIGURE 3 – Intronic restriction site polymorphism in *TβR-I*. (*a*) Exon 7 and flanking sequences of TβR-I from genomic DNA of cervical carcinomas and a BAC genomic clone of *TβR-I* (bac) were amplified by PCR using intronic primers flanking exon 7 and subjected to SSCP. A band with aberrant mobility was detected in 7 of 16 cases (CT51, 52, 58, 60, 63, 65 and 66). The aberrant band was excised from the gel, reamplified by PCR and subjected to direct DNA sequencing. (*b*) Partial DNA sequence of 3' end of TβR-I exon 7 and flanking intron revealed a G→A transition located at 27 nucleotides from the exon-intron boundary. (*c*) The variant sequence results in the loss of a Bsr I restriction site within the intron.

As with TβR-II, we encountered only a single somatic mutation in the TβR-I gene in this series of CCs. This A→C transversion mutation in codon 375 (ACA→ACC) does not alter the amino acid sequence of the protein and is, therefore, silent. Several studies have indicated that sequences that are distant from the classic canonical splice sites can also be involved in splicing regulation (Cooper and Mattox, 1997). However, RT-PCR analysis of RNA from tumor CT65 failed to reveal any splice variants (data not shown). Moreover, even though usage of selective codons in a given mRNA is positively correlated with its translation efficiency,

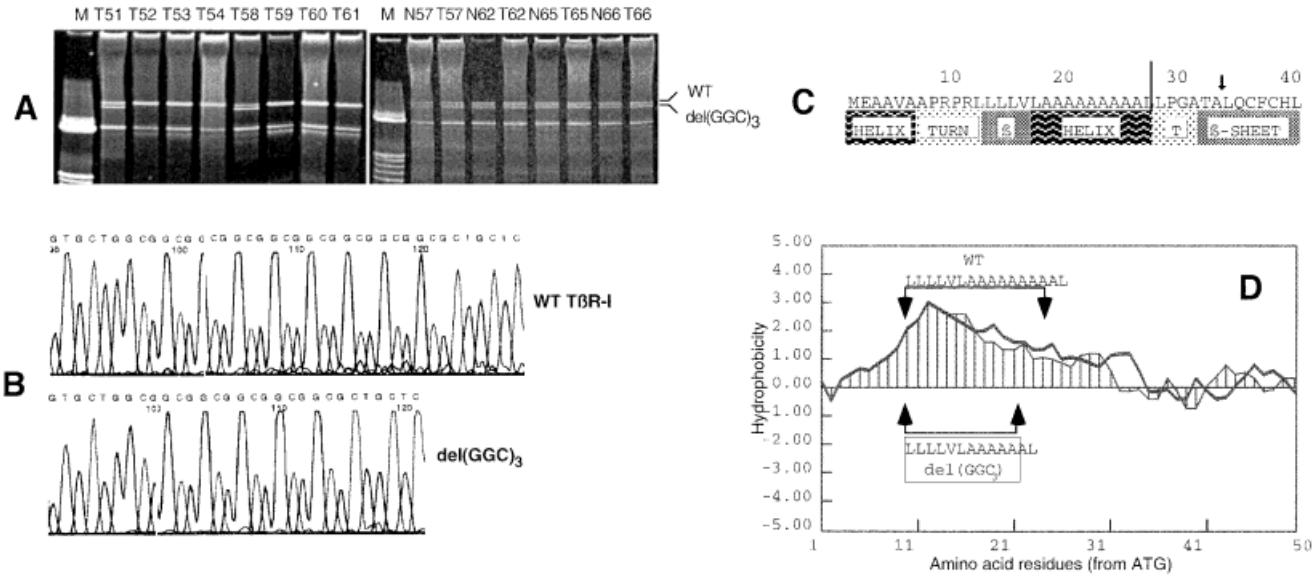

FIGURE 4 – Intragenic deletion within exon 1 of *TβR-I*. (*a*) SSCP analysis of *TβR-I* exon 1. Exon 1 sequences from genomic DNA were amplified by PCR and subjected to SSCP analysis as described in Material and Methods. A band with aberrant mobility corresponding to the del(GGC)3 variant was detected in both germline and tumor DNA of 6 of 16 cases (51, 57, 58, 62, 65 and 66), whereas the remaining cases revealed only the wild type SSCP pattern (cases 52, 53, 54, 59, 60 and 61 shown as examples). T: Tumor. N: Normal tissue. M: Marker lanes. (*b*) Partial DNA sequence of exon 1 of TβR-I revealed an in-frame deletion of 3 of 9 repeating GGC trinucleotides in 6 of 16 cervical carcinomas. (*c*) Predicted secondary structure of the first 40 N-terminal amino acids of *TβR-I* which include the signal sequence using the Chou and Fasman algorithm. The hydrophobic core of the signal sequence consists of an a-helix of 9 alanine residues flanked by 2 Leu residues. ↓ = putative signal peptidase cleavage site. (*d*) Hydrophobicity plot of del(GGC)$_3$ mutant TβR-I predicts for a significant reduction of the hydrophobicity of the putative signal sequence.

this is unlikely to be important in this case, as ACC is the preferred codon for threonine (Kim *et al*., 1997). We have recently described a serine-to-tyrosine substitution (S387Y) within the TβR-I kinase domain in breast cancer specimens (Chen *et al*., 1998). It is interesting that we did not encounter this particular mutation in any of our CC cases. It is possible that the S387Y *TβR-I* mutation is specifically associated with breast cancer. Alternatively, it may be associated most strongly with the metastatic phenotype and not be represented in the present series of CC, most of which were primary tumors.

Besides the single somatic mutation in *TβR-I*, we uncovered 2 interesting polymorphisms in this gene. One of these involves a G→A transition within the intron located between exons 7 and 8. In this case, the variant allele occurs with a frequency of approximately 30% (95% CI: 20–40%), and abolishes a BsrI restriction site. Thus, this restriction site polymorphism will be useful to quantitate the frequency of allelic losses of the TβR-I gene in various human tumors. However, we failed to observe any instances in which the carcinoma was associated with allelic loss of the *TβR-I* gene among the 9 informative cases in the present series (Table I).

The most interesting *TβR-I* polymorphism we detected is an in-frame 9 bp deletion within a GGC trinucleotide repeat sequence located in exon 1. Six of 16 patients with CC were heterozygous carriers of this del(GGC)$_3$ variant allele. More importantly, our data suggest that the del(GGC)$_3$ *TβR-I* variant may be associated with an increased susceptibility to CC. Specifically, we observed twice as many carriers of the del(GGC)$_3$ allele among U.S. patients with CC than among appropriate control subjects (Table II). Interestingly, this relationship was found singularly among U.S. case-control subjects but not among case-control subjects from Jamaica. Since the prevalence of del(GGC)$_3$ carriers was similar among controls in both studies, the difference in results may be due to the small size of each study. However, we cannot exclude a true biological difference between the 2 populations.

Pasche *et al*. (1998) recently reported that the frequency of del(GGC)$_3$ homozygotes was approximately 10 times higher among cancer cases (leukemia, germ cell tumor, colon- and bladder cancer) than among healthy blood donors. Based on these findings, these investigators proposed that the homozygous state is particularly strongly associated with cancer predisposition. The results of our case-control studies confirm this observation: the allele frequency of the del(GGC)$_3$ variant in both control groups was 5% (Table II*c*). Thus, the expected frequency of homozygotes in this population is 0.25%. In fact, the observed frequency of homozygotes was 2.7% among the U.S. cases, and 1.5% overall for both groups of patients with CC, *i.e.*, 5–10 times as high as expected.

The hypothesis that the del(GGC)$_3$ *TβR-I* variant allele confers an increased cancer susceptibility is also biologically plausible. Comerci *et al.* (1996) reported a marked increase in the amount of activated TGFβ associated with stromal cells of invasive CC compared to CIN. This observation suggests that the transformed cervical epithelial cells come under increased selective pressure to escape from TGFβ-mediated control as they progress to CIN to invasive carcinoma. We showed that TβR-I negative cells that express the del(GGC)$_3$ variant are consistently less responsive to TGFβ compared to cells that express wild type receptor. Pasche *et al*. (1998) failed to detect a difference in responsiveness to TGFβ of cells transfected with the del(GGC)$_3$ variant compared to wild type *TβR-I*. However, because of the strong cytomegalovirus promoter used in these experiments, overexpression of both wild type and variant *TβR-I* may well have obscured any subtle difference in membrane expression. This is supported by our experiments in which receptor expression was made limiting by decreasing the amounts of transfected DNA (Fig. 5*b*). The location of the deletion within the putative signal peptide sequence of TβR-I initially suggested that it might affect membrane localization of the mature receptor protein. However, our results indicate that the entire polyalanine repeat region at the N-terminus of TβR-I can be deleted without affecting membrane expression of the protein.

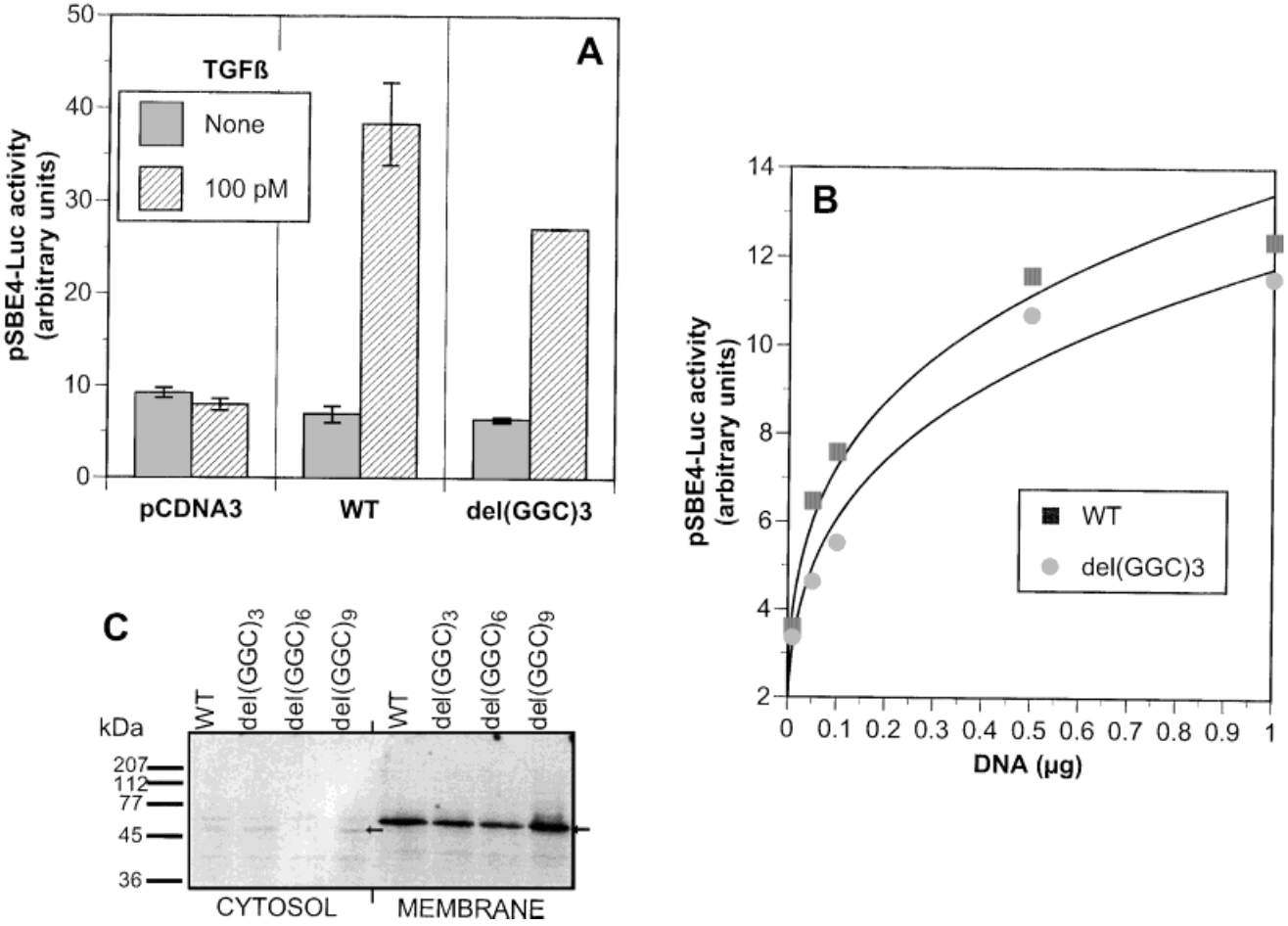

FIGURE 5 – Effects of transfected wild type and del(GGC)3 variant TβR-I receptors on TGFβ-regulated gene expression. (*a*) R-1B (L17) cells were co-transfected with plasmids expressing either wild-type or variant *TβR-I* receptor (1µg) and pSBE4-Luc (2µg) DNA in conjunction with pRL-CMV (0.05µg), and luciferase activities in cell extracts were measured 48 hr later as described in Material and Methods. Results were normalized for *Renilla* luciferase activity to correct for differences in transfection efficiency between experiments. In cells transfected with the *TβR-I* del$(GGC)_3$ variant, human recombinant TGFβ1 (Austral Biologicals, San Ramon, CA) (100 pM) treatment increased SBE4-dependent luciferase activity to a significantly lesser extent than in wild-type *TβR-I* (WT) transfected cells ($p=0.0023$, Student *t*-test). Means ± SEM from 4 independent experiments. (*b*) In order to investigate the relationships between levels of *TβR-I* expression and TGFβ signaling, R-1B (L17) cells were transfected with pSBE4-Luc (1µg) in conjunction with varying amounts of *TβR-I* DNA (range: 0.05–1µg). The differences between wild type and del$(GGC)_3$ variant *TβR-I* induced pSBE4-Luc activity in TGFβ treated cells were greatest when the smallest amounts of *TβR-I* DNA were transfected ($p=0.023$, paired *t*-test). (*c*) Subcellular localization and expression of TβR-I receptors. R1-B (L17) cells were transfected with either full-length *TβR-I* cDNA (WT) or deletion mutants lacking either 3, 6 or all 9 of the GGC repeat units in exon 1 [del$(GGC)_3$, del$(GGC)_6$, or del$(GGC)_9$]. Membrane and cytosolic fractions of transfected cells were isolated as described in Material and Methods and subjected to Western immunoblotting using HA.11 anti-HA antibody. Single discrete 55 kDa bands corresponding to the HA-tagged *TβR-I* receptor were detected in membrane fractions from both wild type- and mutant *TβR-I*-transfected cells, while a slightly faster migrating band of much weaker intensity representing unglycosylated receptor was detectable in the cytosol. Thus, under steady-state conditions, nearly all of the TβR-I receptor protein is found in the cell membrane. Moreover, there were no significant differences between the levels of membrane expression of wild type and any of the 3 deletion mutants of *TβR-I*, indicating that the polyalanine sequence at the N-terminus of the protein is not required for membrane localization of the receptor. Slight differences in intensity among the 4 protein bands paralleled the slight differences in protein loading between lanes, as demonstrated by Ponceau staining of the filters prior to immunoblotting (not shown).

These results imply that the polyalanine region is not part of the signal peptide and may be retained at the N-terminus of the mature peptide. One might speculate, therefore, that this sequence is involved in the formation of TβR-II/TβR-I heterotetramers or that deletions within this region might affect TGFβ binding affinity.

The putative association between the del$(GGC)_3$ allele and CC must be confirmed in a larger study. If correct, this relationship raises the question whether the del$(GGC)_3$ allele affects the susceptibility to persistent infection with high-risk HPV subtypes or the likelihood of progression of early CIN lesions to invasive carcinomas. In either case, such relationships could have important implications for the management of women with cervical dysplasias. Given the fact that one of TGFβ's actions on HPV-infected cells is to inhibit transcription of the viral transforming genes, *E6* and *E7*, it is plausible that HPV infection would favor the clonal expansion of cells that are intrinsically less responsive to TGFβ because they express the del$(GGC)_3$ variant *TβR-I*. We speculate that persistence or regression of CIN lesions may be the result of TGFβ action on cells that are fully responsive, whereas the progression to carcinoma represents the selective outgrowth of clones that are somewhat less responsive to TGFβ, for example, because they express the del$(GGC)_3$ *TβR-I* variant.

In summary, we have identified a number of novel genetic alterations of the *T*β*R-I* and *-II* genes in human invasive CC specimens. Although mutations of either the *T*β*R-I* or *-II* genes appear to represent low frequency events in CC, they provide further support for the hypothesis that inactivation of TGFβ signaling intermediate genes plays a direct role in cancer development. More importantly perhaps, the del$(GGC)_3$ variant allele of the TβR-I gene may be associated with the presence of invasive CC. This observation could have important implications for the control and management of this disease.

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